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Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*

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Six closely related antibacterial proteins, attacins A – F, were isolated from the hemolymph of immunized pupae of the Cecropia moth, *Hyalophora cecropia*. Chromatofocusing separated attacins A – F, with isoelectric points between 5.7 and 8.3. Immunological experiments show that the attacins constitute antibacterially active forms of the previously isolated inducible immune protein P5. Their mol. wts., 20 – 23 K, are similar to that of protein P5, but significantly lower than 28 K found for preP5 synthesized *in vitro* (see accompanying paper). The six attacins can be divided into two groups according to their amino acid composition and amino-terminal sequences, attacins A – D constitute a basic group and attacins E and F an acidic one. Within each group the forms are very similar. The attacins efficiently killed *Escherichia coli* and two other Gram-negative bacteria isolated from the gut of a silk worm but they did not act on other Gram-positive and Gram-negative bacteria tested. Only growing cells of *E. coli* were attacked; cells suspended in phosphate buffer were inert. Besides the cecropins and lysozyme, the attacins represent a third class of antibacterial proteins in the humoral immune system of *H. cecropia*.

Key words: insect immunity/antibacterial proteins/*Hyalophora cecropia*/attacin/immune protein P5

Introduction

Pupae of the Cecropia moth, *Hyalophora cecropia*, respond to an injection of live non-pathogenic bacteria by producing ~15 immune proteins and a potent humoral antibacterial activity (reviewed by Boman and Steiner, 1981). With radioactive amino acids, the immune proteins can be separated by SDS electrophoresis into nine bands, P1 – P9. The two major immune proteins, P4 and P5, have been purified (Rasmuson and Boman, 1979; Pye and Boman, 1977). For none of them was the function elucidated, though P5 was shown to have a synergistic effect when combined with bactericidal ammonium sulphate fractions from the hemolymph (Pye and Boman, 1977).

Much of the antibacterial activity in the hemolymph was traced to the cecropins (first referred to as the P9 proteins by Hultmark *et al.*, 1980), a family of small, basic proteins which have been purified and sequenced (Steiner *et al.*, 1981; Hultmark *et al.*, 1982). Gel filtration of immune hemolymph gives two peaks of antibacterial activity, 'pool I' and 'pool II' of which the latter contains the cecropins and lysozyme (Hultmark *et al.*, 1982). We have now found that the antibacterial factors in pool I represent a set of bactericidal proteins, which are immunologically identical to the previously isolated protein P5. For these proteins we now use the name attacin, from the saturniid tribe *attacini* to which *H. cecropia* belongs. This report summarizes the results obtained in the purification and characterization of the attacins. The accompanying paper describes the isolation and properties of some cDNA clones corresponding to attacins and immune protein P4 (Lee *et al.*, 1983).

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Results

Purification of the attacins

In preliminary experiments, the attacins from pool I were analysed by polyacrylamide gel electrofocusing in combination with an antibacterial assay. Such experiments detected several antibacterial bands. Since the attacins were apparently heterogeneous with respect to isoelectric point, chromatofocusing was employed to isolate the different forms. With a pH gradient from 7.5 to 5, three antibacterial peaks were eluted, one with attacins A – D at the breakthrough of the gradient, one with attacin E at pH 6.8 and a minor peak with the F-form at pH 6.4 (Figure 1).

After chromatofocusing, the polybuffers were removed by precipitation of the attacins with ammonium sulphate at 25% saturation. This step also further improved the purity of the attacins by removing a considerable amount of contaminating protein which remains in solution. The precipitates were dissolved, and desalted by gel filtration as described in Materials and methods.

After ammonium sulphate precipitation, the purity of the attacins was investigated by analytical electrofocusing. The more basic peak with attacins A – D contained at least four different protein bands, focusing between pH 6.4 and 8.3

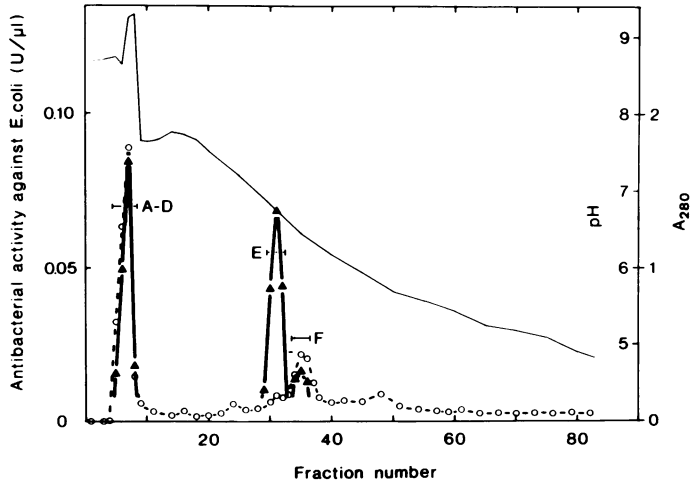

Fig. 1. Chromatofocusing of Pool I at pH 7.5 – 5. Pool I was obtained by gel filtration of immune hemolymph on Sephadex G-100 as described previously (Hultmark *et al.*, 1982). Material corresponding to 5.6 ml hemolymph was chromatofocused on a column (52 x 0.8 cm) of PBE 94, using 300 ml Polybuffer 74 to generate the gradient. The details are given in Materials and methods. Fractions of 4 ml were collected and analysed for antibacterial activity against *E. coli* (▲——▲), absorbance at 280 nm (○——○), and pH (———).

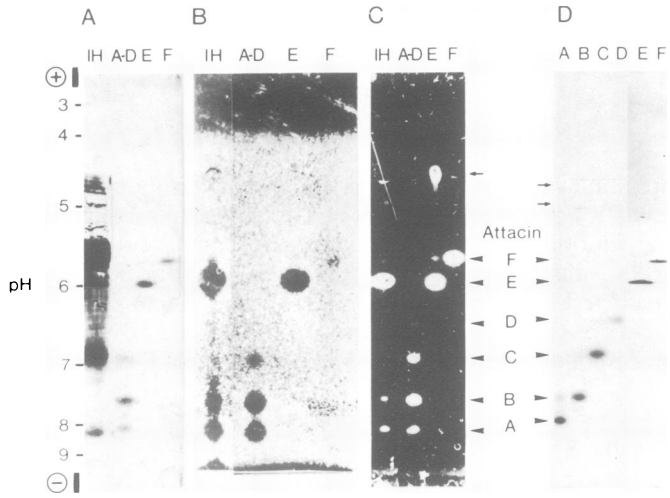

Fig. 2. Analytical electrofocusing of the attacins. The samples were applied directly on the gel surface at positions indicated by small arrows. Sometimes precipitated material remained at these locations. Gels **A** and **D** were stained for protein, gel **B** was overlaid with viable *E. coli* D 31 to detect antibacterial activity, and gel **C** was immunofixated with anti-P5 antiserum. The samples were: in gel **A**, 2 μl of immune hemolymph (IH), 5 μg attacins A–D, 5 μg attacin E, and 3 μg attacin F; in gels **B** and **C** half the amounts of the same samples; in gel **D**, 5 μg purified attacin A and B, 6 μg C, 2 μg D, 5 μg E and 3 μg F.

Table I. Yield and specific activity of the attacins

Sample	Prep. no.[a]	Volume (ml)	Activity (kU_a)	Yield (%)	Specific activity[b] ($U_a/\mu l$)
Pool I	1	5.1	1.86	100	0.04
Attacins A–D	1	3.0	0.22	12	0.20
A	3				0.06
B	3				0.05
C	3				0.03
D	3				0.03
E	1	3.0	0.32	17	0.52
	2				0.24
	3				0.05
F	1	3.0	0.04	2	0.11
	2				0.11
	3				0.04

[a]Results are shown from three different preparations: preparation 1 corresponds to material from 5.6 ml immune hemolymph (c.f., Figure 2). Preparation 2 is from a large preparation, corresponding to 76 ml hemolymph. In preparation 3, the run-through from preparation 2 was re-chromatofocused in order to separate attacins A–D. In this preparation, some attacin E and F was also obtained, probably because of overloading of the column in preparation 2.
[b]The specific activity is the activity in $U_a/\mu l$, divided by the A_{280} of the sample.

(Figure 2A). Each attacin corresponded to one band of antibacterial activity (Figure 2B), though the D form at pH 6.4 was seen only when more material was loaded on the gel (data not shown). Attacins E and F appeared homogeneous on electrofocusing, giving rise to single stained bands. Attacin E corresponded to the major antibacterial band seen at pH 6 after electrofocusing of whole hemolymph. Attacin F focused as a single band with weak antibacterial activity at pH 5.7.

In an attempt to separate attacins A–D in the first peak in Figure 1, this material was re-chromatofocused with a gradient from pH 8.8 to 7 (data not shown). Though these conditions did not give optimal resolution, the four basic components could be isolated in separate peaks. Some cross-contamination occurred between the four forms, but they were essentially free from other contaminating proteins as judged from the stained electrofocusing gel (Figure 2D). The results of the purification of attacins A–F are summarized in Table I.

The six purified attacins were also investigated by SDS-polyacrylamide gel electrophoresis. As shown in Figure 3, they all give bands in the same region, corresponding to mol. wts. of ~22 K for attacin A and B, 20 K for C and D and 23 K for E. Attacin F gave three closely spaced bands, the major one corresponding to a size of 22 K. Apart from the heterogeneity in F and some cross-contamination between the forms, the preparations appeared essentially homogeneous. On occasion a weak band of lower mobility was observed, corresponding to the expected mol. wt. of a dimer. Traces of low mol. wt. peptides were also seen in attacin E.

A problem repeatedly encountered in the purification of the attacins was loss of antibacterial activity, sometimes to levels below detectability. The final concentrated preparations always showed activity, but the specific activities varied (Table I).

Identity with immune protein P5

Using antisera against purified immune protein P5, we tested attacins A–F for cross-reaction. The electrofocusing gel shown in Figure 2C, shows that P5 cross-reacting bands were immunoprecipitated at positions identical to those of attacins A–F. Furthermore, electrofocusing of whole hemo-

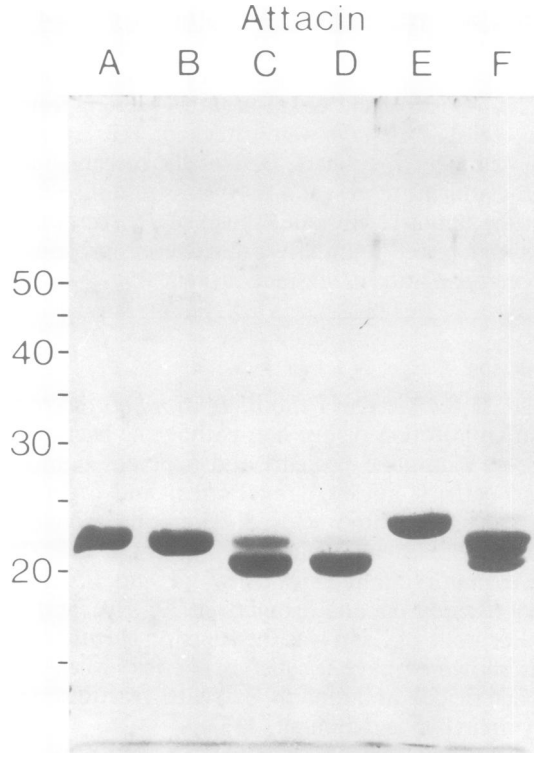

Fig. 3. SDS-polyacrylamide gel electrophoresis of the purified attacins. Attacins A–F, 10 μg of each, were run in 12% polyacrylamide gels. A mol. wt. scale (in K) is indicated. It was determined by running in parallel the following reference proteins: lysozyme (14 388), soybean trypsin inhibitor (21 500), carbonic anhydrase (30 000), alcohol dehydrogenase (41 000) and ovalbumin (45 000).

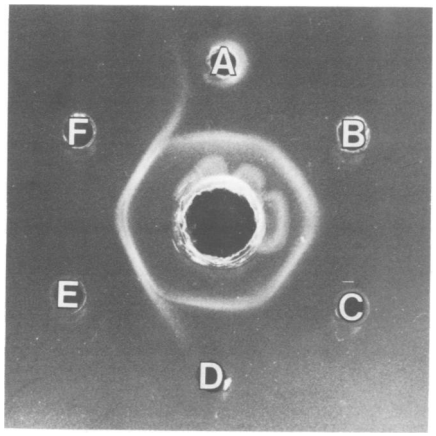

Fig. 4. Ouchterlony double immunodiffusion of the purified attacins against anti-P5 serum. Attacin A–F, 1 μg of each, was added to the outer wells as indicated, and allowed to diffuse against 90 μl of rabbit anti-P5 serum in the central well.

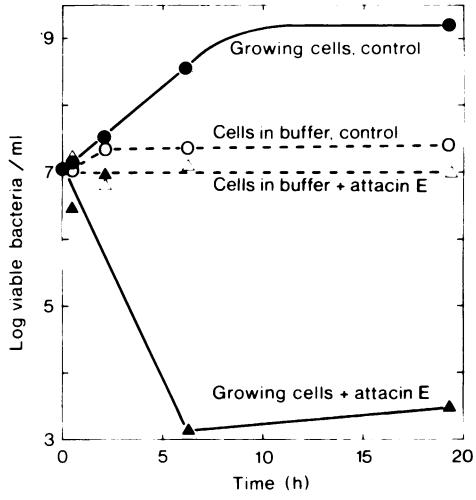

Fig. 5. Effect of attacin on suspensions of *E. coli* D31 (10^7 cells/ml) in either LEG medium or 0.1 M potassium phosphate buffer, pH 6.4. The bacteria were pre-grown at 25°C in LEG medium. Attacin E was added to the suspensions to give a final activity of 20 U_a per ml, and the number of surviving bacteria was estimated after different times of incubation at room temperature (23–26°C) by spreading samples on agar plates. No attacin was added to the controls.

lymph gave rise to the same antibacterial and P5 immunoprecipitation bands as the purified proteins. The conclusion must be that the antibacterial attacins represent most or all of the P5 cross-reacting material in the hemolymph.

The P5 preparation used to immunize the rabbits for the production of antisera was most likely heterogeneous, and thus the reaction of the different forms with the same antisera may not be taken as a proof that they are closely related. However, Ouchterlony double immunodiffusion (Figure 4) shows that an immunologically identical component is present in each of the attacin preparations. In addition, the two acidic attacins E and F give rise to an extra precipitating band, very close to the first one. This band must correspond to an antigenic determinant that is absent in the basic forms.

Antibacterial properties of the attacins

The antibacterial properties of the attacins were in most experiments detected through their capacity to inhibit bacterial growth in solid agar media. Such experiments cannot,

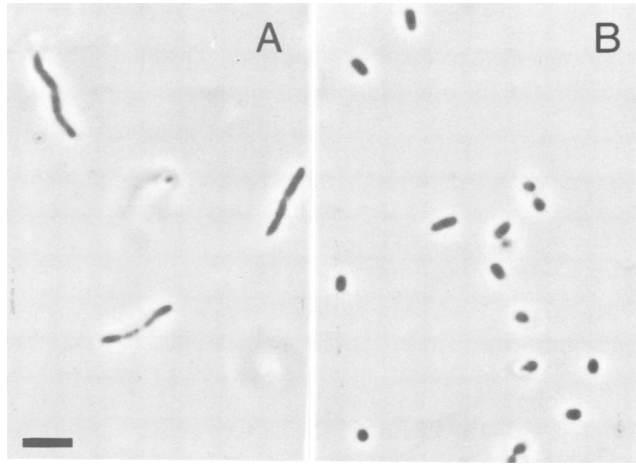

Fig. 6. Phase contrast micrographs of *E. coli* D31 incubated **(A)** with, or **(B)** without attacin. As in Figure 5 the bacteria were incubated for 6 h in LEG medium, with attacin E to a final activity of 13 U_a per ml. The length of the bar corresponds to 5 μm.

Table II. Antibacterial spectra of attacin B and E

Species	Strain	Lethal concentration (μM)	
		Attacin B	Attacin E
E. coli	D21	0.6	2
	D22	0.06	0.3
P. maltophilia	Pm1	4	15
A. calcoaceticus	Ac11	0.4	1
E. cloacae	β12	>15	>20
B. megaterium	Bm11	>15	>20

Lethal concentrations of the attacins were estimated for different bacteria with the inhibition zone assay as described in Materials and methods. *E. cloacae* and *Bacillus megaterium* are two examples of bacteria on which no effect of the attacins was recorded. Other attacin-resistant bacteria were *P. aeruginosa*, strain OT97, *B. subtilis* Bs11, *Streptococcus fecalis* AD-4, *Sarcina lutea* ATCC-9341, and *Micrococcus luteus* Ml11. The agar plates contained LB medium (Bertani, 1951), or, for AD-4, brain heart infusion (Oxoid).

however, discriminate between bactericidal and bacteriostatic effects. In the experiment shown in Figure 5 we have instead added attacin E to a bacterial suspension, and followed the number of surviving bacteria by spreading on agar plates. After a 6 h incubation in a rich medium, permitting bacterial growth, there was a dramatic reduction of the number of colony-forming units. When observed in the phase-contrast microscope, the bacteria were found to form chains, typically containing 4–6 cell-sized units (Figure 6). Some lysis could also be seen, as evidenced by a decreased contrast and by the occurrence of spherical 'blebs' connected to the chains. In the control without attacin, the bacteria were mainly single short rods. In contrast, bacteria incubated in phosphate buffer appeared to be completely resistant to attacin E.

The effect of attacins on different bacteria was compared, using the inhibition zone assay (Table II). Apart from *Escherichia coli*, the only attacin-sensitive organisms were two Gram-negative bacteria, *Acinetobacter calcoaceticus* and *Pseudomonas maltophilia*, both isolated from the gut of a Chinese oak silkworm. The chain forming *envA* mutant, D22, a division-defective strain, was considerably more sensitive than the parental strain, *E. coli* D21. The inhibition

zones obtained with attacins have a diffuse border while the cecropins give sharp and well defined zones.

As shown in Figure 7, the attacins are rather heat-stable, with 40% of the antibacterial activity remaining after 1 h at 100°C. Furthermore, in electrofocusing the antibacterial band of attacin E remained in the same position after heating (Figure 7, insert). Only a minor part of the protein was modified by the harsh treatment, giving rise to an extra band with a slightly higher pI.

Molecular properties of the attacins

The amino acid composition was determined for each of the six attacins (Table III). These data indicate that attacins A–D form one group, and attacins E and F a second. Within each group, the amino acid composition of the different forms is virtually identical, the differences being within the limits of the experimental errors. Furthermore, the two groups are clearly related to each other, though significant differences exist. All attacins have a very high content of aspartic acid, much glycine and alanine and unusual levels of phenylalanine and threonine. Together these amino acids contribute more than half of the residues. The ratio of aspartic to glutamic acid, and of lysine to arginine, is very high. The basic attacins A–D have slightly higher levels of threonine, glutamic acid, lysine and tryptophan, while the acidic forms E and F have more aspartic acid, isoleucine and arginine. With protein concentration determined from the amino acid composition, the absorption coefficients (A_{280}, 1 mg/ml, 1 cm) were found to be 0.8–1.0 for attacins A–D and 0.4–0.5 for the E and F forms.

Automatic Edman sequencing of the purified proteins gave the amino-terminal sequences for all forms except attacin D, of which too little material was available. The sequences shown in Figure 8 give further evidence of a close relationship between the attacins. No difference was seen between the basic forms, A–C, or between the acidic forms, E and F. Among the 20 residues determined for the basic attacins A–C, there were only three amino acid exchanges compared with the two acidic forms E and F. In addition, the latter contained two extra residues in the N terminus.

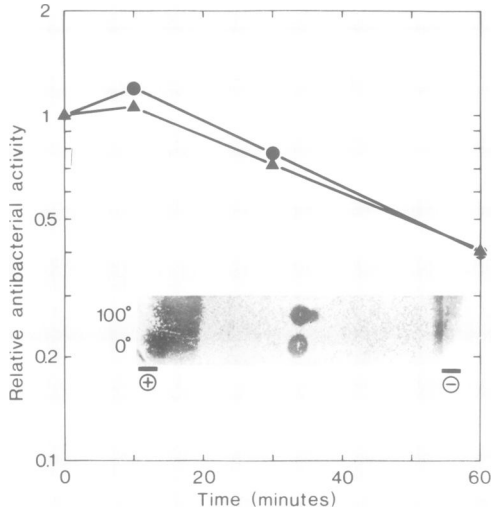

Fig. 7. Heat stability of attacin activity. Small samples of attacin B (●) and E (▲) were incubated at 100°C as described in Materials and methods. The remaining antibacterial activity was assayed against *E. coli* D31, using the inhibition zone assay. Inset shows electrofocusing of attacin E, incubated at 0 or 100°C. The gel was seeded with live *E. coli* D31 to visualize the antibacterial activity.

Discussion

With gel filtration, one or two steps of chromatofocusing and ammonium sulphate precipitation, attacins A–F were isolated, all of them antibacterial and cross-reacting with P5

Table III. Amino acid composition of the attacins

Amino acid	Residues per 100 total residues; Attacin					
	A	B	C	D	E	F
Asp[a]	16.4	16.2	16.3	16.6	19.6	19.9
Thr	9.1	9.1	9.2	9.1	7.5	7.7
Ser	7.4	7.4	7.4	7.5	7.3	6.9
Glu[a]	2.8	2.9	3.1	3.3	1.4	1.5
Pro	4.2	4.2	4.5	4.2	3.7	3.8
Gly	9.7	9.7	9.8	9.5	9.9	10.2
Ala	10.7	10.8	10.7	10.8	10.8	11.0
Cys	–	–	–	–	–	–
Val	4.7	4.7	4.7	4.6	4.7	4.8
Met	1.1	0.6	1.3	1.3	1.1	1.5
Ile	3.4	3.3	3.2	3.4	5.2	5.5
Leu	6.0	6.0	6.0	6.1	6.0	6.2
Tyr	2.5	2.5	2.2	2.0	1.9	1.2
Phe	8.6	8.7	8.4	8.3	8.1	7.2
His	3.1	3.2	3.2	3.2	3.1	3.1
Lys	8.6	8.6	8.3	8.2	7.7	7.2
Arg	0.7	0.6	0.6	0.8	1.6	1.6
Trp[c]	1.0	1.4	1.1	1.2	0.6	0.5

[a]Including the corresponding amide.
[b]Determined after oxidation to cysteic acid for attacin F only.
[c]Tryptophan was determined spectrophotometrically.

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                          1                   5                  10
Attacin  A - C            Ala-Gly-Ala-Leu-Thr-Ile-Asn-Ser-Asp-Gly-Thr-Ser-Gly-

Attacin  E - F  Asp-Ala-His-Gly-Ala-Leu-Thr-Leu-Asn-Ser-Asp-Gly-Thr-Ser-Gly-
                 1                   5                  10                  15

                      15                  20
Attacin  A - C  Ala-Val-Val-Lys-Val-Pro-Ile-

Attacin  E - F  Ala-Val-Val-Lys-Val-Pro-Phe-Ala-Gly-Asn-Asp-Lys-Asn-Ile-
                                     20                  25
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Fig. 8. Amino-terminal structure of the attacins as determined by automatic Edman sequencing. The differences between the two forms are boxed.

antibodies. The mol. wts. of the attacins, 20 – 23 K (Figure 3), are close to the previously published value of 24 K for the monomer of P5 (Pye and Boman, 1977). However, it is significantly lower than the 28 K found for P5 cross-reacting material synthesized *in vitro* (Lee *et al.*, 1983). This could be due to processing of the precursor(s) of the attacins. In addition, a partial nicking may have occurred near the C terminus in attacin F. This form gave three bands in SDS-electrophoresis (Figure 3), in spite of the fact that its N-terminal structure appeared homogeneous. The similarity in amino acid composition to attacin E also argues against major contamination with unrelated proteins.

Besides lysozyme and the cecropins, the attacins represent a third class of inducible, antibacterial, immune proteins in the hemolymph of *H. cecropia*. The two acidic forms of attacin may be related to the acidic antibacterial proteins earlier observed in other moths (Stephens and Marshall, 1962; Croizier, 1979). Immunological cross-reactivity in combination with electrofocusing (as in Figure 2) may possibly settle the relationship.

The loss of antibacterial activity during the purification of the attacins remains unexplained. Since it is not correlated with the appearance of new bands in electrophoresis or electrofocusing, it is unlikely that the loss of activity is due to major changes in primary structure. Furthermore, the heat stability of the attacins indicates that conformational changes are not easily introduced. One remaining possibility is that the activity is associated with a cofactor that is lost during the purification. We have, however, been unable to reconstitute the activity by recombining fractions after separation. Loss of antibacterial activity, and the fact that activity was only assayed with non-growing cells, may explain why Pye and Boman (1977) did not detect the antibacterial properties of P5.

The amino-terminal sequences of the attacins have two tetrapeptides, Ser-Asp-Gly-Thr, and Val-Val-Lys-Val, in common with the DNA-damaging anti-tumor antibiotic neocarzinostatin (NCS) from *Streptomyces carzinostaticus* (Meienhofer *et al.*, 1978). They occur as one continuous sequence in NCS, but are interrupted by three residues in the attacins. If these peptides are aligned, homology is found in 10 of the 29 residues determined for the acidic attacins, NCS having four additional residues at the N terminus. The activity of NCS is associated with a low mol. wt. chromophore (Napier *et al.*, 1981). For attacins a cofactor is also suspected but their normal protein spectra give no evidence of this (results not given). Moreover, NCS is about half the size of the attacins, and it has a different amino acid composition. Therefore, at present, no firm conclusion can be reached about the significance of the observed homology.

Under the conditions tested, the attacins proved to be effective only against a few Gram-negative bacteria. This narrow spectrum is in contrast to cecropin A and B which act on a variety of both Gram-positive and Gram-negative bacteria. However, the mechanisms of action may be totally different; the cecropins cause destruction of bacterial membranes (Steiner *et al.*, 1981), while the limited data presented here indicate that attacins only act on growing cells and cause chain formation (Figures 5 and 6). The fact that our chain-forming *envA* mutant D22 was considerably more sensitive than its parental strain can be taken as support for the hypothesis that the target(s) of the attacins is in the cell division cycle. Since strain D22 is also barrier defective (Boman *et al.*, 1971), an alternative explanation to its sensitivity would be that its outer membrane shows an increased permeability to attacins.

The six attacins form two groups, the basic A – D and the acidic E – F, which cross-react immunologically. All attacins have similar mol. wts. (Figure 3), antibacterial activities (Table II), amino acid compositions (Table III) and closely related N-terminal structures (Figure 8). The acidic attacins are distinguished by the presence of an extra band in the double diffusion against P5 antibodies (Figure 4). This may indicate either the presence of two antigenically different forms or possibly two different antigenic determinants on the same molecule (Clausen, 1969), only one of which is also present in the basic attacins. To explain all these data one needs to assume only two very similar attacin genes, originating from a gene duplication. Since cDNA clones for attacins have been isolated (Lee *et al.*, 1983), a combination of protein chemistry and molecular genetics may in the future settle both the gene organization of the attacins and the primary structures of the multiple forms.

Materials and methods

The insects, preparation of immune hemolymph

Diapausing pupae of *H. cecropia* were obtained commercially, or reared in the laboratory (Hultmark *et al.*, 1980), and stored at 8°C. They were immunized with living *Enterobacter cloacae*, β12, and hemolymph was collected after 7 days at 25°C, as described (Hultmark *et al.*, 1980).

Antibacterial assays

The antibacterial assay used is a modification of Flemings classical method. Inhibition zones were recorded around wells in thin agar plates with bacteria as described (Hultmark *et al.*, 1982). To calculate lethal concentrations the diameters *(d)* of the inhibition zones were recorded for different amounts of attacin *(n)* in the well. Plotting d^2 against log n, a linear relation was obtained, and the lethal concentration (c_l) could be calculated from the slope *(k)* and intercept *(l)* according to the formula:

$$c_l = \frac{4 \ln 10}{\pi a k 10^{l/k}} = \frac{2.93}{a k 10^{l/k}}$$

where a is the thickness of the agar layer. If a and d are given in cm, and n in nmol, c_l will be expressed in μM. Very small zones deviate from the linear relation, and for these the lethal concentration can be calculated directly from the zone diameter:

$$c_l = \frac{4n}{\pi e a d^2} = \frac{0.468 n}{a d^2}$$

where e is the base of the natural logarithms. The derivation of these expressions is given in Hultmark *et al.* (1982) though the first expression contains a misleading printer's error. One absolute unit of activity (U_a) is the amount of antibacterial activity in 1 ml of sample at the lethal concentration. It should be noted that the c_l obtained can depend on the media used (Sidén and Boman, 1983). In Table II the medium was LB (Bertani, 1951); in all other experiments LB was supplemented with medium E (Vogel and Bonner, 1956) and 0.2% glucose (LEG medium).

Purification of the attacins

Pool I (see Figure 2 in Hultmark *et al.*, 1982) was isolated from immune hemolymph by gel filtration on a Sephadex G-100 column equilibrated with 0.15 M ammonium acetate, pH 5, and 0.01% phenylthiourea. Pool I, identified by its antibacterial activity, was well separated from the cecropins and from the bulk of the hemolymph proteins.

The isolated Pool I was freeze-dried for several days to remove all ammonium acetate, then dissolved in Polybuffer 74 (Pharmacia Fine Chemicals AB, Uppsala, Sweden), pH 5, diluted 1:10 with water. The material was applied to a chromatofocusing column packed with PBE 94 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and equilibrated with 0.025 M Tris-HCl buffer, pH 8. The column was eluted with Polybuffer 74, 1:10, pH 5, generating a gradient between pH 7.5 and 5. The attacins E and F eluted in separate peaks, while attacin A, B, C and D eluted in a single peak.

In one experiment, the material containing attacins A – D was pooled and applied to a second column with PBE 94, equilibrated with 0.025 methanol-amine-HCl, pH 10. The column was eluted with Polybuffer 96-HCl, 1:10, pH 7, generating a pH gradient from pH 8.8 to 7. Attacins A – D eluted as

closely spaced peaks near the beginning of the gradient.

As an alternative to chromatofocusing, the attacins were also purified by ion-exchange chromatography. Pool I, dialysed against 0.03 M ammonium acetate, pH 5.1, was applied to a column, packed with CM-Sepharose (Pharmacia Fine Chemicals AB, Uppsala, Sweden), and equilibrated with the same buffer. The attacins were eluted with a gradient of ammonium acetate, 0.03–0.5 M. Attacin F used for N-terminal sequencing and SDS-electrophoresis was prepared in this way. Amino acid composition and behaviour in electrofocusing was identical for attacin F prepared both ways.

In either method, the peak fractions that contained the different attacins were identified by their antibacterial activity, and/or by double immunodiffusion with P5 antibodies, and they were pooled separately. The attacins were precipitated by the addition of 0.144 g ammonium sulphate/ml sample, giving 25% saturation, and dissolved in a small amount of water. For amino acid analysis, the remaining ammonium sulphate was removed in a PD-10 desalting column (Pharmacia Fine Chemicals AB, Uppsala, Sweden), equilibrated with 0.05 M ammonium formate. Water and ammonium formate was then removed by extensive freeze-drying.

Bacteria used

The standard test organism used for antibacterial assays was *E. coli* D31. *E. coli* D22 is a chain-forming, *envA* mutant derived from strain D21. The pedigree and properties of these three strains are given by Boman *et al.* (1971). *P. maltophilia* strain Pm1 and the parental strain of *A. calcoaceticus* Ac11 were both isolated simultaneously from the gut of a healthy, prestarved larva of the Chinese oak silkmoth, *Antheraea pernyi*, collected at Guangxi Agricultural College, Nanning, China. Strain Ac11 is a spontaneous streptomycin resistant mutant of the original isolate. References to other strains are given by Hultmark *et al.* (1982).

Immunological techniques

For immunization of rabbits protein P5 was prepared according to Pye and Boman (1977), but omitting the electrofocusing step. It gave a single band in SDS-electrophoresis, but probably contained several of the attacin forms. Rabbits were injected i.m. with 80–100 μg P5 in Freund's complete adjuvant. Three injections were given at 1 month intervals, and blood was collected from an ear vein 2 weeks after the last injection, and then at weekly intervals. Every second week boosters of 30–40 μg P5 were given. Ouchterlony double immunodiffusion was carried out according to Clausen (1969).

Electrofocusing and electrophoresis

Electrofocusing was performed on LKB ready-made ampholine PAG-plates, pH 3.5–9.5 (LKB-Produkter AB, Bromma, Sweden) essentially according to the instructions from the manufacturer. To localize bands with antibacterial activity, the gels were washed for 30 min in buffered bacterial medium and then overlaid with *E. coli* D31 in agar as previously described (Hultmark *et al.*, 1980). The top agar layer was omitted. After incubation overnight at 37°C, antibacterial activity in the bands was visualized as areas without bacterial growth. Specific immunofixation of attacin bands was carried out on gels, washed for 30 min in 0.2 M potassium phosphate, pH 7.4, by pouring antiserum directly on top of the gel and incubating overnight. The precipitates could be photographed directly, or after washing in 0.9% NaCl and water.

SDS-electrophoresis was carried out in 1 mm thick, 105 mm long, 12% polyacrylamide gels, using a discontinuous SDS-Tris-glycine buffer system as described by Weber and Osborn (1975). The samples were pretreated and stained as previously described (Hultmark *et al.*, 1980).

Heat treatment

Tightly sealed 1.5 ml plastic tubes containing attacin samples (5 μl) in 0.05 M ammonium formate were transferred from an ice bath to an empty bottle immersed in a covered boiling water bath. After different times they were transferred back to the ice bath. The tubes were centrifuged for 5 min at 10 000 *g*, mixed, and then centrifuged again to recover any moisture that had condensed on the walls. The supernatants were assayed for antibacterial activity with the inhibition zone assay.

Amino acid analysis and sequencing

The details for these procedures were as previously (Hultmark *et al.*, 1982). Tryptophan was estimated from the absorbance at 280 nm, after correcting for the presence of tyrosine. Contributions from other amino acids were neglected. The molar absorptivity of tryptophan was taken to be 5600, and for tyrosine 1197/M/cm (Sober, 1970).

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